

# *Farnesoid X receptor and liver X receptor ligands initiate formation of coated platelets*

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# Farnesoid X Receptor and Liver X Receptor Ligands Initiate Formation of Coated Platelets

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**Objectives**—The liver X receptors (LXRs) and farnesoid X receptor (FXR) have been identified in human platelets. Ligands of these receptors have been shown to have nongenomic inhibitory effects on platelet activation by platelet agonists. This, however, seems contradictory with the platelet hyper-reactivity that is associated with several pathological conditions that are associated with increased circulating levels of molecules that are LXR and FXR ligands, such as hyperlipidemia, type 2 diabetes mellitus, and obesity.

**Approach and Results**—We, therefore, investigated whether ligands for the LXR and FXR receptors were capable of priming platelets to the activated state without stimulation by platelet agonists. Treatment of platelets with ligands for LXR and FXR converted platelets to the procoagulant state, with increases in phosphatidylserine exposure, platelet swelling, reduced membrane integrity, depolarization of the mitochondrial membrane, and microparticle release observed. Additionally, platelets also displayed features associated with coated platelets such as P-selectin exposure, fibrinogen binding, fibrin generation that is supported by increased serine protease activity, and inhibition of integrin  $\alpha$ IIB $\beta$ 3. LXR and FXR ligand-induced formation of coated platelets was found to be dependent on both reactive oxygen species and intracellular calcium mobilization, and for FXR ligands, this process was found to be dependent on cyclophilin D.

**Conclusions**—We conclude that treatment with LXR and FXR ligands initiates coated platelet formation, which is thought to support coagulation but results in desensitization to platelet stimuli through inhibition of  $\alpha$ IIB $\beta$ 3 consistent with their ability to inhibit platelet function and stable thrombus formation in vivo.

**Visual Overview**—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2017;37:1482-1493. DOI: 10.1161/ATVBAHA.117.309135.)

**Key Words:** bile ■ blood coagulation ■ blood platelets ■ calcium ■ cholesterol

Platelets act as the first line of defense after vascular injury and play a key role in the prevention of excessive blood loss through the formation of a thrombus at the site of vessel damage. Unwanted, excessive platelet activation and hyper-reactive platelets are thought to contribute to atherothrombosis that can lead to myocardial infarction and stroke. Several pathological conditions such as hyperlipidemia,<sup>1,2</sup> type 2 diabetes mellitus,<sup>3,4</sup> metabolic syndrome,<sup>5</sup> obesity,<sup>5,6</sup> and high cholesterol<sup>7-12</sup> have been related to platelet hyper-reactivity and increased platelet responses.

Formation of procoagulant platelets is one way in which platelets can be classed as hyper-reactive. A subclass of procoagulant platelets are coated platelets (previously called COAT platelets)<sup>13</sup> that expose and retain high levels of phosphatidylserine and high levels of  $\alpha$ -granule proteins on their surface including factor V, fibrinogen, fibronectin, and von Willebrand

factor.<sup>14-16</sup> Coated platelets are also associated with deregulated levels of intracellular calcium, depolarized mitochondrial membranes, generation of reactive oxygen species (ROS), loss of membrane integrity, and the release of platelet microparticles.<sup>17-19</sup> Coated platelets are reported to support coagulation reactions, whereas cleavage and inactivation of proteins required for integrin signaling prevents aggregation and inhibits traditional platelet activation in response to platelet agonists.<sup>20,21</sup>

Several intracellular nuclear receptors that have been identified in human platelets such as the liver X receptor (LXR) and farnesoid X receptor (FXR), are recognized for their roles in the transcriptional regulation of metabolic pathways that are disrupted in conditions such as type II diabetes mellitus, metabolic syndrome, and hyperlipidemia.<sup>22-27</sup> The LXR receptors are involved in the regulation of cholesterol homeostasis

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**The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.117.309135/-/DC1>.**

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### Nonstandard Abbreviations and Acronyms

<b>FXR</b>	farnesoid X receptor
<b>GPVI</b>	glycoprotein VI
<b>LXR</b>	liver X receptor
<b>ROS</b>	reactive oxygen species

and their natural ligands, for example, oxysterols, are cholesterol derivatives.<sup>28–33</sup> Cholesterol derivatives have been found to deregulate platelet responses to several platelet agonists, with some derivatives potentiating and others inhibiting aggregation.<sup>34–36</sup> Similarly, the FXR receptor is activated by bile acids and other cholesterol derivatives with roles in the regulation of bile acid and cholesterol homeostasis<sup>37–39</sup> and has also been shown to play a role in insulin homeostasis.<sup>40,41</sup> Bile acids have previously been associated with platelet dysfunction<sup>42</sup> and treatment of platelets with bile acids has been shown to be associated with membrane vesiculation and platelet swelling.<sup>43,44</sup>

Increasing evidence supports nongenomic actions for intracellular nuclear receptors, including FXR and LXR, that are clearly distinct from their well-established genomic roles.<sup>27,45,46</sup> Previous work has shown that ligands for both LXR and FXR inhibit platelet activation by GPVI (glycoprotein VI) agonists and thrombin, as evidenced by inhibition of platelet aggregation, granule secretion, and calcium mobilization in response to collagen receptor GPVI agonists and thrombin. Furthermore outside-in signaling through integrin  $\alpha\text{IIb}\beta 3$  is inhibited resulting in a significant reduction in thrombus formation and stability in vivo,<sup>25,27</sup> although the mechanisms by which these ligands cause this inhibition seem to be different.<sup>25,27</sup>

The ability of LXR and FXR ligands to inhibit platelet function seems at odds with the observations that conditions associated with increased circulating levels of LXR or FXR ligands, such as hyperlipidemia, type 2 diabetes mellitus, metabolic syndrome, obesity, and high cholesterol are associated with increased platelet reactivity and an increased risk of cardiovascular disease.<sup>1–12</sup> Furthermore, many natural and synthetic LXR and FXR ligands including hydroxycholesterols and bile acids have been shown to be cytotoxic and proapoptotic in some cell types.<sup>47–53</sup> We have also previously observed that platelets treated with high concentrations of FXR ligand GW4064 showed decreased sample turbidity indicative of platelet swelling and procoagulant activity.<sup>27</sup> Therefore, despite previous observations that treatment of platelets with LXR or FXR ligands inhibits platelet function to platelet agonists, understanding how these nuclear receptor ligands nongenomically affect platelets before agonist stimulation is of clear importance.

It has been previously described that during thrombus formation 2 distinct populations of platelets appear, coaggregated platelets, which support thrombus growth, and loosely attached platelets that expose phosphatidylserine, which support coagulation but cannot support platelet activation.<sup>20,54</sup> We set out to determine whether LXR and FXR ligands can both activate and inhibit platelets.

In this study, we sought to explore in more detail how LXR and FXR ligands may affect the ability of platelets to respond to platelet agonists. We identify that treatment of platelets with LXR or FXR ligands can convert platelets to a procoagulant state that is consistent with coated platelet formation.

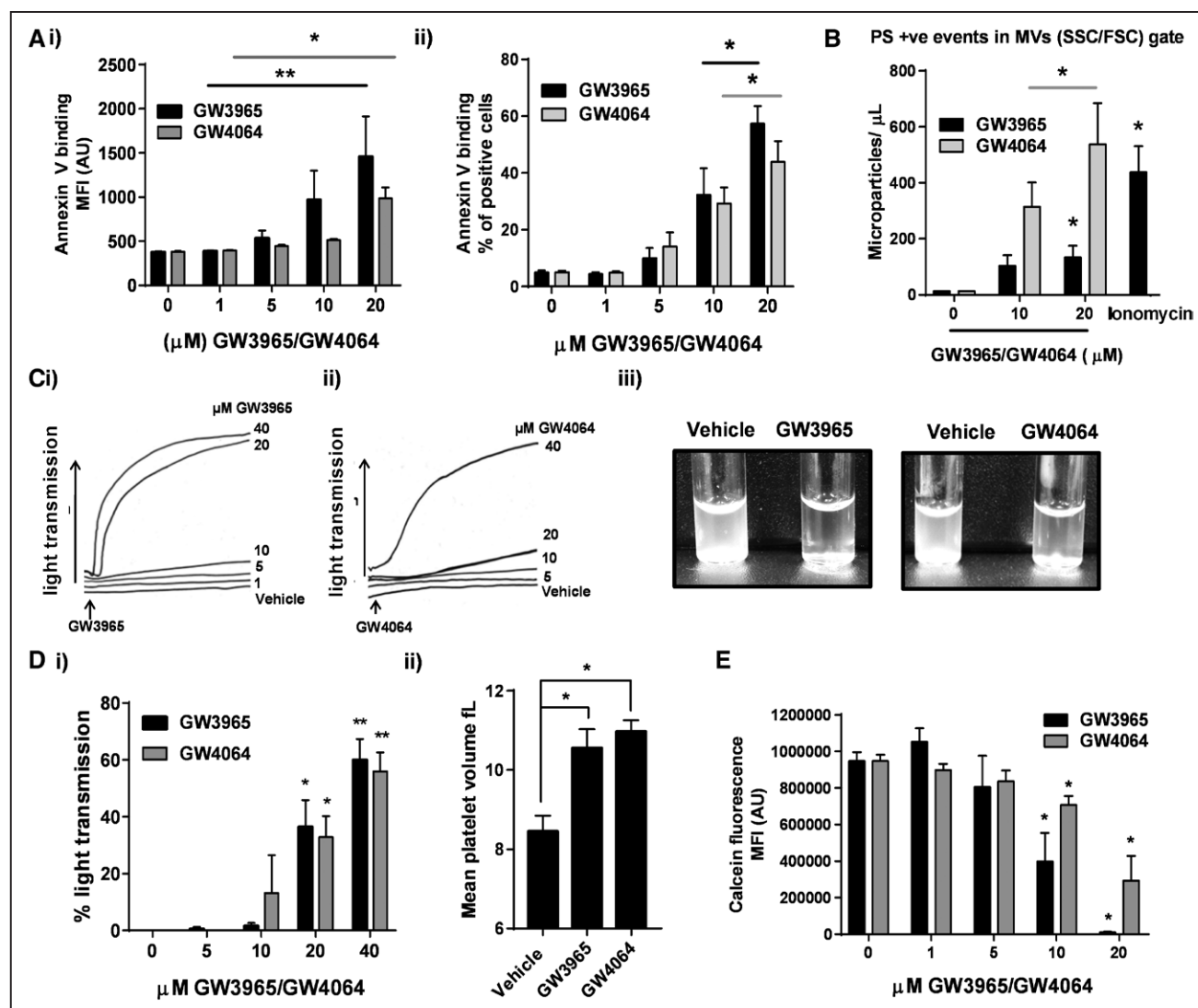
## Materials and Methods

Materials and Methods are available in the [online-only Data Supplement](#).

## Results

### LXR and FXR Ligands Increase Procoagulant Activity of Platelets

We set out to determine whether treatment with the LXR or FXR ligands (using concentrations previously shown to inhibit platelet function) was able to convert resting platelets to the procoagulant state. To do this, several markers of procoagulant activity were measured, including phosphatidylserine exposure, microparticle formation, platelet swelling, and loss of membrane integrity. Phosphatidylserine exposure was determined by measuring annexin V binding after treatment of washed platelets with either the LXR ligand GW3965 or FXR ligand GW4064 (1, 5, 10, and 20  $\mu\text{mol/L}$ ) for 10 minutes. Platelets treated with either GW3965 or GW4064 showed a concentration-dependent increase in phosphatidylserine exposure with increases in the median fluorescence intensity (Figure 1Ai) and percentage of positive cells (Figure 1Aii) for annexin V binding observed (gating strategy shown in Figure 1A in the [online-only Data Supplement](#)). Microparticle formation from washed platelets (Figure 1B) was also increased in a concentration-dependent manner in GW3965- or GW4064-treated platelets in comparison to vehicle-treated controls (gating strategy shown in Figure 1B through 1D in the [online-only Data Supplement](#)). Platelet swelling (Figure 1C and 1D), measured as a decrease in sample turbidity and an increase of mean platelet volume (using an ACT5 hematology analyzer) was also evident after treatment with either the LXR or FXR ligands (5, 10, 20, and 40  $\mu\text{mol/L}$ ). The observed decrease in sample turbidity was not because of aggregation as it could not be prevented by pretreatment with integrilin (5  $\mu\text{mol/L}$ , a concentration shown to block fibrinogen binding and aggregation), and cell lysis was excluded as treatment with GW3965 or GW4064 did not reduce platelet count (Figure 2 in the [online-only Data Supplement](#)).<sup>20</sup> Finally, membrane integrity (Figure 1E) determined by measuring calcein permeability was also altered after treatment with either GW3965 or GW4064. Both compounds caused a decrease in calcein fluorescence, indicating that the integrity of the platelet membrane had been compromised, as has been previously observed during procoagulant platelet formation.<sup>21,55,56</sup> In support of these observations being the result of specific activity of the LXR and FXR receptors, concentration-dependent increases in annexin V binding were also observed after treatment with natural ligands for the LXR receptor 27-hydroxycholesterol and 24S-hydroxycholesterol (10, 30, and 100  $\mu\text{mol/L}$ ) or natural ligand for the FXR receptor chenodeoxycholic acid (100 and 300  $\mu\text{mol/L}$ ; Figure 3 in the [online-only Data Supplement](#)).



**Figure 1.** Liver X receptor (LXR) and farnesoid X receptor (FXR) ligands induce platelet procoagulant activity. Human washed platelets were treated for 10 min with or without increasing concentrations of GW3965 (1, 5, 10, 20, and 40  $\mu\text{mol/L}$ ) or GW4064 (1, 5, 10, 20, and 40  $\mu\text{mol/L}$ ) or vehicle control before analysis by flow cytometry for (A) annexin V binding, which is a measure of phosphatidylserine exposure, and data expressed as (i) median fluorescence intensity and (ii) percentage of annexin V-positive cells; (B) formation of microparticles, determined by gating for the microvesicle population using forward and side scatter profiles of ApogeeMix beads. Data expressed as number of phosphatidylserine (PS)-positive events per microliter. C and D, Platelet swelling, measured by analyzing (C) light transmission, with an increase in transmission associated with increased platelet swelling (i) and (ii) representative traces and (iii) representative images. D, (i) data expressed as % of light transmission; (ii) mean platelet volume. E, Calcein fluorescence, to measure membrane integrity, expressed as median fluorescence intensity. Results are mean+SEM for  $n \geq 3$ . \* $P < 0.05$  in comparison to vehicle controls.

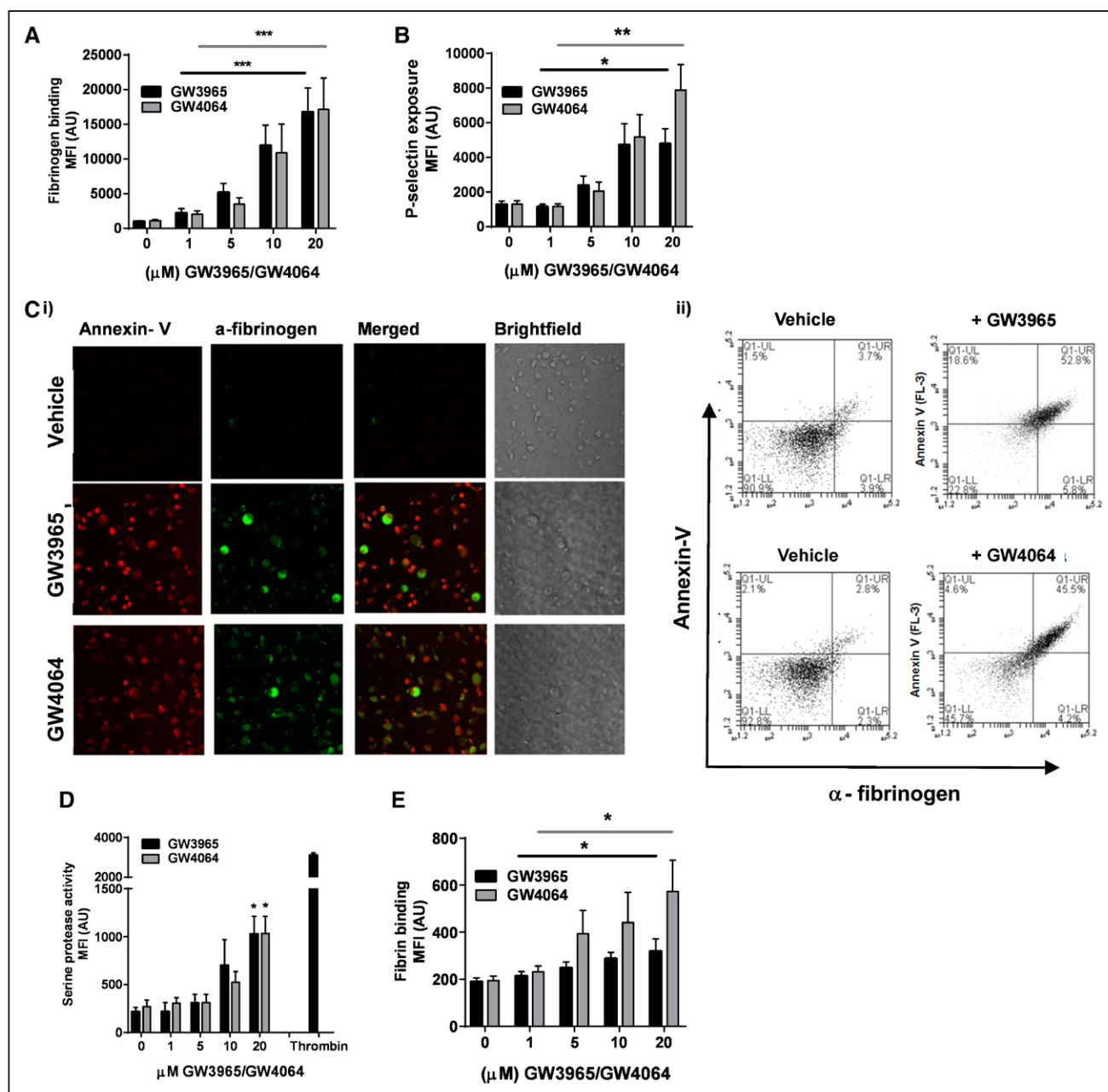
### LXR and FXR Ligands Initiate Formation of Coated Platelets

Coated platelets are procoagulant platelets that in addition to exposure of phosphatidylserine are characterized by high levels of surface retention and exposure of  $\alpha$ -granule proteins including factor V, fibrinogen, fibronectin, and von Willebrand factor. To determine whether GW3965 and GW4064 were capable of stimulating coated platelet formation, fibrinogen binding, and P-selectin exposure were measured after treatment with either ligand. Washed platelets treated with either the LXR or FXR ligand (1, 5, 10, and 20  $\mu\text{mol/L}$ ) exhibited a concentration-dependent increase in fibrinogen binding and P-selectin exposure in comparison to vehicle-treated controls (Figure 2A and 2B). Because no

exogenous fibrinogen was added to the assay, we hypothesize that the fibrinogen bound at the platelet surface comes from the activated platelet via  $\alpha$ -granule secretion, which also leads to increased P-selectin exposure at the platelet surface. As with annexin V binding, treatment of platelets with the natural ligands for the LXR and FXR receptors, 27-hydroxycholesterol and 24S-hydroxycholesterol (10, 30, and 100  $\mu\text{mol/L}$ ) or chenodeoxycholic acid (100 and 300  $\mu\text{mol/L}$ ) also caused an increase in fibrinogen binding, further supporting that these observations are because of specific activity of the LXR and FXR receptors (Figure III in the [online-only Data Supplement](#)).

In further support of procoagulant coated platelet formation, immunofluorescence microscopy of platelets stained





**Figure 2.** Liver X receptor (LXR) and farnesoid X receptor (FXR) agonists initiate coated platelet formation. Human washed platelets were treated with increasing concentrations of GW3965 (1, 5, 10, and 20  $\mu$ M/L), GW4064 (1, 5, 10, and 20  $\mu$ M/L), or vehicle control and tested for several different markers of coated platelet formation including (A) fibrinogen binding, (B)  $\alpha$ -granule components, measured by detecting P-selectin exposure, (D) serine protease activity, measured by quantification of release of fluorescence as a result of conversion of Z-Gly-Gly-Arg-AMC fluorescent substrate for 1 h at 37°C, and (E) bound fibrin. Data expressed as median fluorescence intensity. C, Human platelets treated with GW3965 (20  $\mu$ M/L), GW4064 (20  $\mu$ M/L), or vehicle were stained with annexin V-Cy5 (in red) and anti-fibrinogen (Alexa 488 conjugated; in green) and analyzed by (i) fluorescence microscopy on a  $\times 100$  oil immersion lens and (ii) flow cytometry. (i) Representative images and (ii) representative scatter plots shown. Other results are mean+SEM for  $n \geq 3$ . \* $P < 0.05$  in comparison to vehicle controls.

with annexin V and an antifibrinogen antibody confirmed the formation of phosphatidylserine exposing ballooned platelets that bind fibrinogen after treatment with GW3965 (20  $\mu$ M/L) and GW4064 (20  $\mu$ M/L) in comparison to vehicle-treated controls (Figure 2Ci). In further support of this, imaging flow cytometry confirmed that GW3965- and GW4064-treated platelets show an increase in fibrinogen and annexin V binding compared with vehicle-treated

control (Figure IV in the [online-only Data Supplement](#)), and single cell flow cytometry 2 feature dot plot analysis showed that platelets found to be positive for annexin V binding were also found to bind fibrinogen. This is shown in Figure 2Cii, with an increased number of events observed in the upper right quadrant of the dot plot after treatment with GW3965 or GW4064 compared with vehicle control (Figure 2Cii).

Increases in platelet prothrombinase activity and the subsequent conversion of fibrinogen into fibrin is another key feature of coated procoagulant platelets.<sup>57</sup> The ability of the LXR and FXR agonists to induce platelet prothrombinase activity was quantified as the rate of cleavage of Z-Gly-Gly-Arg-AMC, a serine protease substrate. Both GW3965- and GW4064-treated platelets showed a significant increase in serine protease activity in comparison to vehicle controls (Figure 2D). Although this is likely to reflect assembly of the prothrombinase complex at the cell surface, the action of other coagulation enzymes may also contribute. In support of this increase in serine protease activity being because of increased thrombin activity, a dose-dependent increase in surface fibrin, detected using an antifibrin antibody, was also observed after treatment with either GW3965 or GW4064 (Figure 2E). Additionally, analysis of thrombin generation in platelet-rich plasma by calibrated automated thrombography found that treatment with either GW3965 or GW4064 reduced the time taken to reach peak thrombin generation compared with vehicle-treated controls (Figure V in the [online-only Data Supplement](#)). Together, these observations support a role for both LXR and FXR ligands in the regulation of platelet prothrombinase (serine protease) activity and fibrin generation.

### LXR and FXR Ligand-Dependent Formation of Procoagulant Platelets Inhibits Activation of Integrin $\alpha$ IIb $\beta$ 3

It has been shown that fibrinogen binding to coated platelets is independent of activation of integrin  $\alpha$ IIb $\beta$ 3.<sup>16,20</sup> We, therefore, tested whether the observed increase in fibrinogen binding in GW3965- or GW4064-treated platelets was associated with activation of integrin  $\alpha$ IIb $\beta$ 3. In support of integrin  $\alpha$ IIb $\beta$ 3 not being activated, neither GW3965 nor GW4064 caused an increase in PAC-1 antibody binding to the open (active) conformation of integrin  $\alpha$ IIb $\beta$ 3 (Figure 3A). Furthermore, pretreatment of platelets with integrilin at a concentration (5  $\mu$ mol/L) known to block fibrinogen binding to integrin  $\alpha$ IIb $\beta$ 3<sup>27</sup> was unable to reverse the observed increase in fibrinogen binding (Figure 3B). These results suggest that increased binding of fibrinogen to the platelet surface is independent of integrin  $\alpha$ IIb $\beta$ 3 activation as has been previously described for coated platelet formation.<sup>16,20</sup>

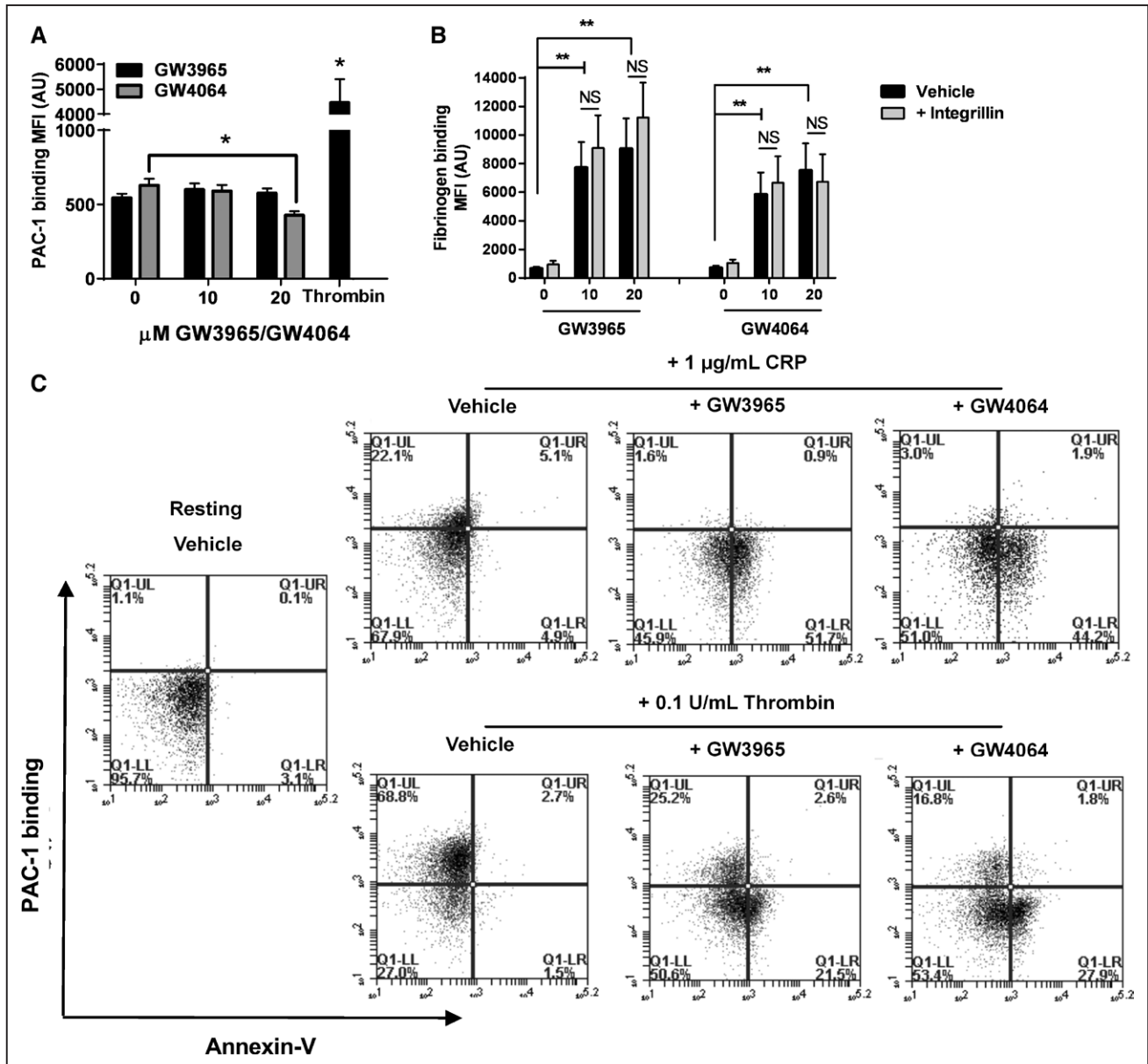
Phosphatidylserine exposure in coated platelets is associated with closure and inhibition of the  $\alpha$ IIb $\beta$ 3 integrin and other platelet receptors.<sup>20</sup> Interestingly, previously published data have shown that treatment with similar concentrations of GW3965 and GW4064 causes inhibition of platelet aggregation to GPVI agonists and thrombin.<sup>25,27</sup> In further support of these findings, we observed inhibition of integrin  $\alpha$ IIb $\beta$ 3 function, determined by measuring PAC-1 binding, after treatment with GW3965 and GW4064 (10 and 20  $\mu$ mol/L) and stimulation with collagen-related peptide (CRP-XL; 1  $\mu$ g/mL) or thrombin (0.1 U/mL; Figure VIA in the [online-only Data Supplement](#)). Treatment with GW3965 and GW4064 also resulted in reduced cell adhesion and spreading on fibrinogen-coated coverslips (Figure VIB and

VIC in the [online-only Data Supplement](#)). In support of the formation of coated platelets after treatment with GW3965 and GW4064 leading to inhibition of integrin  $\alpha$ IIb $\beta$ 3, dot plot analysis of CRP-stimulated (1  $\mu$ g/mL) and thrombin-stimulated (0.1 U/mL) platelets shows an increase in phosphatidylserine exposure and a reduction in PAC-1 binding after treatment with GW3965 or GW4064 compared with vehicle controls. In particular, platelets identified as positive for annexin V binding were found to be negative for PAC-1 binding, as shown by lack of events in the upper right quadrant of the dot plot (Figure 3C). Analysis of nonadhered platelets after exposure to fibrinogen-coated coverslips for 45 minutes also showed that GW3965- and GW4064-treated platelets displayed significantly increased levels of annexin V binding in the nonadhered platelets (which is associated with inhibition of adhesion and spreading) compared with vehicle control (Figure VID in the [online-only Data Supplement](#)). Finally, we tested whether the observed increase in fibrinogen binding in GW3965- or GW4064-treated platelets was associated with Talin cleavage, a calcium-dependent step in the activation of integrin  $\alpha$ IIb $\beta$ 3. As shown in Figure VIE in the [online-only Data Supplement](#), platelets treated with GW3965 (20  $\mu$ mol/L) or GW4064 (20  $\mu$ mol/L) did not show cleavage of the Talin head domain compared with an ionomycin-treated (5  $\mu$ mol/L) positive control, further suggesting that integrin  $\alpha$ IIb $\beta$ 3 is not activated, leaving  $\alpha$ IIb $\beta$ 3 unable to initiate outside-in signaling.

### GW3965- and GW4064-Induced Platelet Procoagulant Activity Is Associated With Depolarization of the Mitochondrial Membrane and Is Dependent on Sustained Increases in Intracellular Calcium

Phospholipid scrambling and conversion to the procoagulant state in platelets is attributed to both loss of mitochondrial transmembrane potential and an increase in intracellular calcium.<sup>19,55</sup> To test whether this was the underlying mechanism behind the observed characteristics of procoagulant platelets induced by the LXR and FXR ligands, the integrity of the mitochondrial membrane was investigated using the JC-1 fluorophore. As shown in Figure 4A in comparison to vehicle control, both GW3965 and GW4064 cause a significant reduction in the JC-1 FL2/FL1 ratio, indicating mitochondrial membrane depolarization. It has also been previously described that cyclophilin D can play a role in formation of procoagulant platelets after stimulation by platelet agonists.<sup>19</sup> Treatment of platelets with cyclosporine A, a cyclophilin D inhibitor, prevented FXR ligand GW4064-mediated increases in annexin V binding similar to that observed in thrombin- and CRP-stimulated platelets, but phosphatidylserine exposure after treatment with LXR ligand GW3965 was unaffected similar to that observed after treatment with ionomycin (Figure 4B). Thereby identifying a role for cyclophilin D in FXR agonist but not in LXR agonist mediated increases in platelet procoagulant activity.

Single platelet analysis of calcium signaling, looking at immobilized individual platelets under arterial shear flow rates (20 dyn/cm<sup>2</sup> that is equivalent to 1000 s<sup>-1</sup>) identified that when



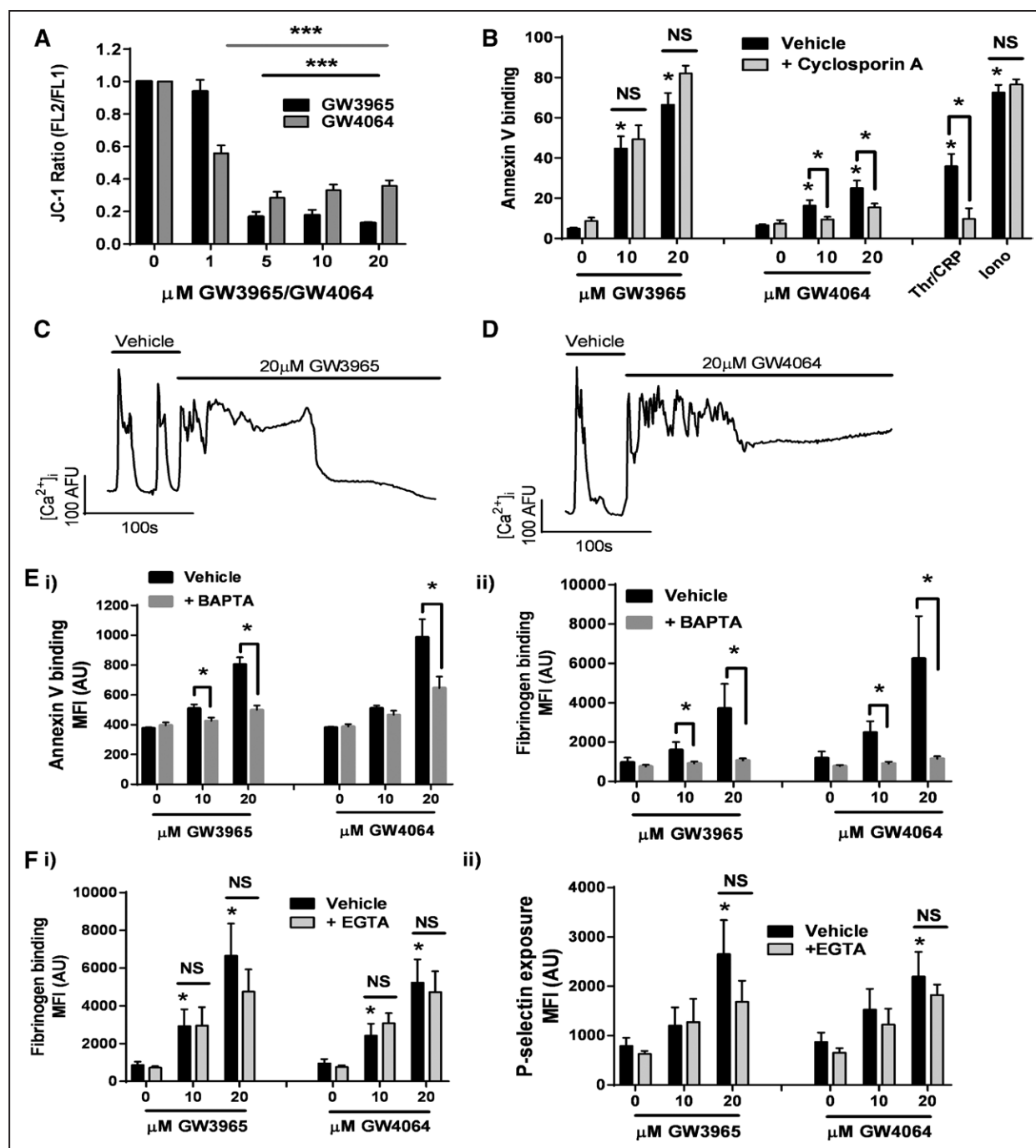
**Figure 3.** Liver X receptor (LXR) and farnesoid X receptor (FXR) agonists inhibit  $\alpha\text{IIb}\beta_3$ . Human washed platelets were prepared, and the effect of GW3965 or GW4064 (10 and 20  $\mu\text{mol/L}$ ) on (A) integrin  $\alpha\text{IIb}\beta_3$  activation determined by PAC-1 antibody binding and (B) fibrinogen binding in the presence or absence of integrillin (5  $\mu\text{mol/L}$ ), an inhibitor of integrin  $\alpha\text{IIb}\beta_3$  fibrinogen binding. (Data expressed as median fluorescence intensity, mean+SEM for  $n \geq 3$ ). C, PAC-1 binding and phosphatidylserine exposure in CRP-stimulated (1  $\mu\text{g/mL}$ ) and thrombin-stimulated (0.1 U/mL) platelets was determined. Representative scatter plots shown.

treating platelets with vehicle control, spikes of calcium signaling (intermittent pulses of fluorescence) are observed that are likely to be because of the shear forces the platelets experience under flow.<sup>58</sup> In contrast, when flowing either GW3965 or GW4064 over the platelets, single platelet calcium signaling was increased and sustained compared with the signaling observed in the presence of vehicle control (Figure 4C and 4D). This increased and sustained signaling is then seen to dissipate over time, which appears to be because of the compromised integrity of the platelet membrane and leakage of the calcium dye from the platelet.

Having identified alterations in calcium signaling after treatment with either LXR or FXR ligands, we set out to

determine whether the observed formation of coated platelets was dependent on intracellular or extracellular calcium. Platelets were treated with either GW3965 or GW4064 or vehicle in the presence or absence of BAPTA-AM (10  $\mu\text{mol/L}$ ) to chelate intracellular calcium (but leaving extracellular calcium levels unaffected) or EGTA (1 mmol/L), which chelates extracellular calcium. Treatment of platelets with BAPTA-AM prevented the GW3965- and GW4064-induced increases in annexin V binding and fibrinogen binding even after treatment with high concentrations of either ligand, suggesting that the observed phosphatidylserine exposure is dependent on intracellular calcium (Figure 4E). In contrast, treatment of platelets with EGTA did not prevent GW3965- and GW4064-induced





**Figure 4.** Mitochondrial transmembrane potential and sustained calcium signaling are required for the induction of LXR- and FXR-mediated procoagulant activity. Human washed platelets were treated for 10 min with or without increasing concentrations of GW3965 (1, 5, 10, and 20  $\mu\text{M}$ /L) or GW4064 (1, 5, 10, and 20  $\mu\text{M}$ /L) or vehicle control before analysis by flow cytometry for (A) changes in the mitochondrial membrane potential, determined by using JC-1 dye and expressing the results using the FL2/FL1 ratio. An increase in the ratio compared with control indicates hyperpolarization of the mitochondrial membrane, whereas a decrease in the FL2/FL1 ratio indicates membrane depolarization. B, Platelets were treated for 10 min with or without increasing concentrations of GW3965 (10 and 20  $\mu\text{M}$ /L) or GW4064 (10 and 20  $\mu\text{M}$ /L) or vehicle control in the presence of cyclosporine A (5  $\mu\text{M}$ /L), a cyclophilin D inhibitor, before analysis by flow cytometry for annexin V binding. Data expressed as percentage of annexin V-positive events. Thrombin-treated (0.1 U/mL)/CRP-treated (1  $\mu\text{g}$ /mL) and ionomycin-treated (5  $\mu\text{M}$ /L) samples were included as positive controls. C and D,  $\text{Ca}^{2+}$  imaging in single platelets under flow. Human washed platelets loaded with Fluo4-AM (2  $\mu\text{M}$ /L) were attached onto mouse anti-human PECAM-1 antibody-coated (WM59) glass-bottom Vena8 GCS biochips (Cellix Ltd, Dublin, Ireland) and vehicle. C, GW3965 (20  $\mu\text{M}$ /L) or (D) GW4064 (20  $\mu\text{M}$ /L) were then flowed through the chips at a slow shear rate of 400  $\text{s}^{-1}$  and calcium signaling monitored by observing fluorescence for 5 min. Single platelet, representative calcium signaling trace shown. E and F, Platelets were treated with GW3965 or GW4064 (0, 10, and 20  $\mu\text{M}$ /L) in the presence or absence of (E) BAPTA (10  $\mu\text{M}$ /L) or (F) EGTA (1 mmol/L) before analysis by flow cytometry for (E) annexin V binding, and fibrinogen binding and (F) fibrinogen binding and P-selectin exposure. Thr (0.1 U/mL) and Thr (0.1 U/mL)+CRP (5  $\mu\text{g}$ /mL) included as positive controls for fibrinogen binding in the presence of EGTA. Data expressed as median fluorescence intensity. Results are mean+SEM for  $n \geq 3$  and expressed as percentage of vehicle control. \* $P < 0.05$  in comparison to vehicle controls.

increases in fibrinogen binding or P-selectin exposure (annexin V binding not monitored, as annexin V requires presence of  $\text{Ca}^{2+}$  in the buffer to bind phosphatidylserine), suggesting that LXR and FXR ligand-induced formation of coated platelets is not dependent on extracellular calcium (Figure 4F).

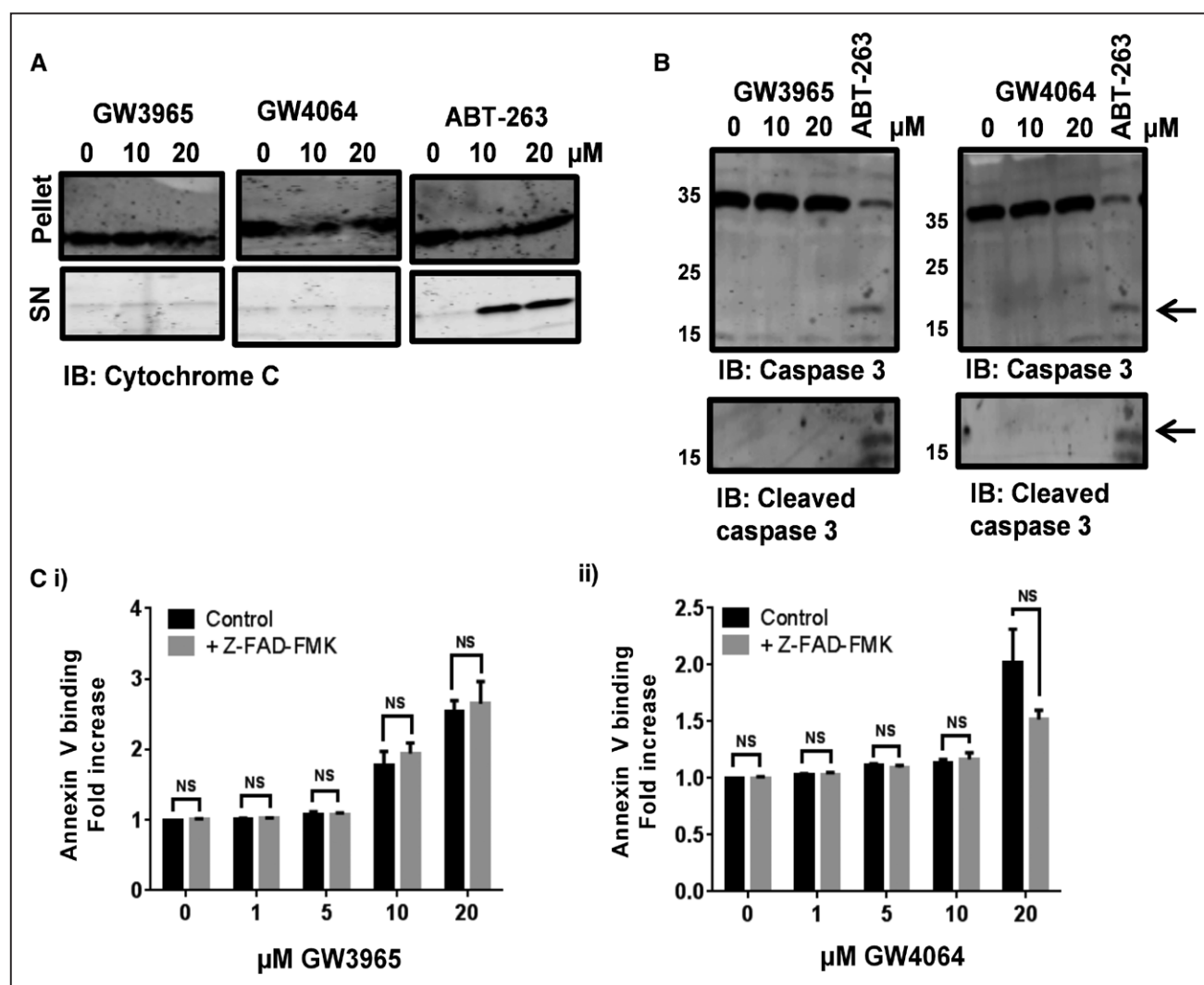
### GW3965 and GW4064 Do Not Activate Apoptotic Pathways

In other cell types, FXR and LXR agonists have been shown to cause cytotoxicity and initiation of apoptosis.<sup>47–53</sup> As several of the observed changes induced by GW3965 and GW4064, such as phosphatidylserine exposure and depolarization of the mitochondrial membrane potential, are also observed during cellular apoptosis, the effects of GW3965 and GW4064 on cytochrome c release and caspase activation were determined. As shown in

Figure 5, neither was capable of causing the cytosolic release of cytochrome c (a key stage before caspase activation that occurs in apoptosis) or caspase cleavage, in contrast to ABT-263 (10–20  $\mu\text{mol/L}$ ), an initiator of apoptosis. This was confirmed by incubation of platelets with a caspase inhibitor Z-FAD-FMK (10  $\mu\text{mol/L}$ ) before treatment with GW3965 or GW4064 as Z-FAD-FMK was unable to reverse the potentiation of annexin V binding (Figure 5C), suggesting the observations described here are because of conversion of platelets to the procoagulant state and not a result of initiation of cellular apoptosis.

### LXR and FXR Ligand-Induced Procoagulant Activity Is Dependent on Reactive Oxygen Species

The generation of reactive oxygen species (ROS) is associated with and dependent on coated platelet formation.<sup>18,19</sup> To



**Figure 5.** Liver X receptor (LXR) and farnesoid X receptor (FXR) ligands do not initiate apoptosis. **A** and **B**, Human washed platelets were pretreated with GW3965 or GW4064 (10 and 20  $\mu\text{mol/L}$ ) or ABT-263 (10 and 20  $\mu\text{mol/L}$ ) for 2 h before **(A)** lysis in mitochondria isolation lysis buffer. The heavy mitochondrial membrane was then isolated from its releasate by centrifugation, and the presence of cytochrome C in the mitochondrial releasate determined by Western blotting. **B**, Activation and cleavage of caspases was also determined in GW3965-treated or GW4064-treated (10 and 20  $\mu\text{mol/L}$ ) platelet lysates by Western blotting for the cleaved activated form of the caspases. ABT-263 (20  $\mu\text{mol/L}$ ) treated platelets were included as a positive control for initiation of apoptosis. **C**, Annexin V binding was determined in **(i)** GW3965-treated and **(ii)** GW4064-treated platelets after pretreatment with a caspase inhibitor Z-FAD-FMK (10  $\mu\text{mol/L}$ ). Representative blots are shown, and results are mean+SEM for  $n=3$  and expressed as fold increase compared with control. \* $P<0.05$  in comparison to vehicle controls.

determine whether the mode of action of the LXR and FXR ligands in converting platelets to coated platelets involves ROS generation, the ability of GW3965 and GW4064 to initiate formation of ROS was determined using the fluorescent dye H2DCFDA. Small increases in H2DCFDA fluorescence and ROS generation were observed after treatment with either GW3965 or GW4064 (20  $\mu\text{mol/L}$ ; Figure 6A). It has previously been described that antioxidants can prevent Thr/CRP-mediated formation of coated platelets,<sup>18</sup> and in support of a role for ROS in GW3965- and GW4064-mediated increases in procoagulant coated platelet formation, we found that phosphatidylserine exposure and fibrinogen binding were significantly attenuated in the presence of a ROS scavenger *n,n'*-diphenyl-*p*-phenylenediamine (Figure 6B and 6C). These data suggest an important role for ROS in LXR and FXR ligand-dependent coated platelet formation. Given the involvement of ROS but not components of the platelet apoptotic pathways, this suggests that platelets are converted to the procoagulant state via a pathway similar to necrosis, as described previously.<sup>55</sup>

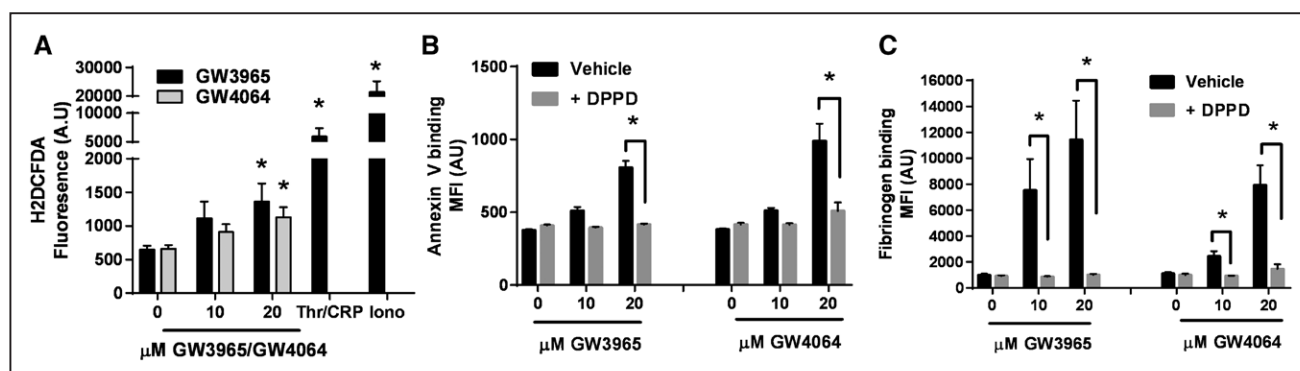
## Discussion

Here, we describe that both LXR and FXR receptor ligands are capable of converting platelets to the procoagulant state at micromolar concentrations that cause inhibition of platelet activity and show receptor specificity in knockout mouse models as previously reported.<sup>25,27</sup> The effects of the ligands presented here are likely to be physiologically relevant as endogenous LXR and FXR ligands can circulate in the plasma at micromolar concentrations under certain conditions and localized concentrations could be much higher. In a healthy individual, total cholesterol levels (LXR ligands) are maintained <4 mmol/L, but significantly higher levels have been reported in several conditions. Normal levels of bile acids (FXR ligands) in the systemic circulation are reported to be  $\geq 10$   $\mu\text{mol/L}$  in postprandial conditions, which can be further increased in disease.<sup>59</sup>

The data presented here are consistent with studies that have found that cholesterol derivatives, LXR receptor ligands,

deregulate platelet responses to several platelet agonists<sup>34–36</sup> and that treatment with bile acids can cause platelet dysfunction,<sup>42</sup> including membrane vesiculation and platelet swelling.<sup>43,44</sup> Treatment with either LXR or FXR ligands caused a concentration-dependent increase in phosphatidylserine exposure that was associated with microparticle formation and platelet swelling/ballooning. Increased mean platelet volume is a known marker of platelet hyper-reactivity and associated with increased risk of thrombosis. Increased mean platelet volume has also been observed in several pathological conditions including hyperlipidemia, obesity, high cholesterol, and type 2 diabetes mellitus,<sup>60–63</sup> conditions associated with increased circulating levels of hydroxycholesterols and bile acids (LXR and FXR agonists). Our findings could, therefore, offer some explanation behind these observations. It is also interesting to note that these observations after treatment with GW3965 and GW4064 also provide a novel example of platelet ballooning and swelling that occurs independently of stimulation by traditional platelet agonists collagen and thrombin.<sup>56</sup>

Concentration-dependent increases in fibrinogen binding (that is not associated with integrin  $\alpha\text{IIb}\beta 3$  activation),  $\alpha$ -granule protein exposure at the platelet surface and increased fibrin generation were also observed after treatment with LXR and FXR agonists, indicating the formation of a subset of procoagulant platelets known as coated platelets that are thought to be involved in supporting coagulation reactions. Similar to other previously described examples of increases in procoagulant activity and coated platelet formation, LXR and FXR agonist-dependent increases in procoagulant activity were found to be associated with, and dependent on, sustained intracellular calcium signaling, ROS, and loss of mitochondrial transmembrane potential, all of which have previously been described to be required for Thr/CRP-dependent coated platelet formation.<sup>13,19,20,64,65</sup> Interestingly, one difference was observed with cyclophilin D, which was found to play a role in FXR agonist-mediated procoagulant platelet formation but was not required for LXR agonist-mediated coated platelet formation.



**Figure 6.** Generation of reactive oxygen species (ROS) is required for induction of procoagulant platelet activity by liver X receptor (LXR) and farnesoid X receptor (FXR) ligands. Human washed platelets were treated for 10 min with or without increasing concentrations of GW3965 (10 and 20  $\mu\text{mol/L}$ ) or GW4064 (10 and 20  $\mu\text{mol/L}$ ; **A**) in the presence of the fluorescent dye H2DCFDA (an indicator of ROS) and fluorescence measured. Thrombin-treated, CRP-treated, and ionomycin-treated platelets were included as positive controls. **B** and **C**, In the presence or absence of 10  $\mu\text{mol/L}$  DPPD (*n,n'*-diphenyl-*p*-phenylenediamine), a reactive oxygen species scavenger before analysis by flow cytometry for **(B)** annexin V binding and **(C)** fibrinogen binding, using an antifibrinogen antibody. Results are mean+SEM for  $n \geq 3$  and expressed as median fluorescence intensity. \* $P < 0.05$  in comparison to vehicle controls.

Despite appearing to be hyper-reactive when in the procoagulant state and able to support the assembly of coagulation complexes that lead to fibrin generation, coated platelets have been shown to undergo closure of integrin  $\alpha\text{IIb}\beta_3$  and other platelet receptors,<sup>20</sup> reducing the responsiveness of these platelets to platelet stimuli. Coated platelets, therefore, show a significant reduction in their ability to be activated by platelet agonists.<sup>55</sup> Previous studies have shown that FXR and LXR ligands inhibit platelet aggregation to GPVI agonists and thrombin,<sup>25,27</sup> and this is further supported by our observations that PAC-1 binding is reduced after treatment with either GW3965 or GW4064 in both CRP- and thrombin-stimulated platelets. It is particularly important to highlight that it is those platelets found to be positive for annexin V binding that show significantly reduced PAC-1 binding compared with vehicle controls, thereby suggesting that procoagulant coated platelet formation is associated with reduced integrin  $\alpha\text{IIb}\beta_3$  activation. Previously published data describe that agonists for both the LXR and FXR receptors attenuate platelet responses to stimulation by both GPVI agonists and thrombin and also inhibit stable thrombus formation after arterial laser injury in vivo.<sup>25,27</sup> It has been described that during thrombus formation, 2 distinct populations of platelets appear, coaggregated platelets with activated  $\alpha\text{IIb}\beta_3$  integrins, which support thrombus growth, and loosely attached platelets that expose phosphatidylserine, which support coagulation but cannot support platelet activation.<sup>20</sup> We hypothesize that it is this reduction in integrin  $\alpha\text{IIb}\beta_3$  activity observed on coated platelet formation after treatment with either GW3965 or GW4064 that underlies this inhibition of thrombus formation in vivo. We propose that exposure of platelets to LXR and FXR ligands convert platelets to this procoagulant coated state, yet despite their ability to support coagulation, these platelets are desensitized to platelet stimuli through inhibition of integrin  $\alpha\text{IIb}\beta_3$ , thereby appearing to negatively regulate platelet functional responses. It is also interesting to note that procoagulant platelets and platelets with increased platelet volume have been shown to be more rapidly cleared from the circulation. This could lead to thrombocytopenia if platelet production is not balanced with platelet turnover,<sup>55,66</sup> although further study would be required to determine whether this is the case after prolonged exposure to either LXR or FXR ligands.

The work described here has significant clinical implications as circulating and localized concentrations of several agonists for these nuclear receptors, including cholesterol derivatives and bile acids, are increased under several pathophysiological conditions. These nongenomic effects of nuclear receptor ligands could, therefore, contribute to platelet deregulation. Ligands for LXR and FXR are under clinical development for the treatment of diabetes mellitus, high cholesterol, and obesity; and it is therefore important to balance their beneficial metabolic effects against their possible side effects on platelet reactivity and function.

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### Disclosures

None.

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### Highlights

- Treatment of platelets with liver X receptor or farnesoid X receptor ligands converts platelets to the procoagulant state and forms coated platelets.
- Liver X receptor and farnesoid X receptor ligand-induced coated platelets display increased phosphatidylserine exposure, platelet swelling, microparticle formation, increased P-selectin exposure, fibrinogen binding, and fibrin generation that is supported by increased serine protease activity.
- These coated platelets are, however, desensitized to platelet stimuli through receptor closure and cleavage, which is consistent with their ability to inhibit platelet function and stable thrombus formation in vivo.

# Arteriosclerosis, Thrombosis, and Vascular Biology



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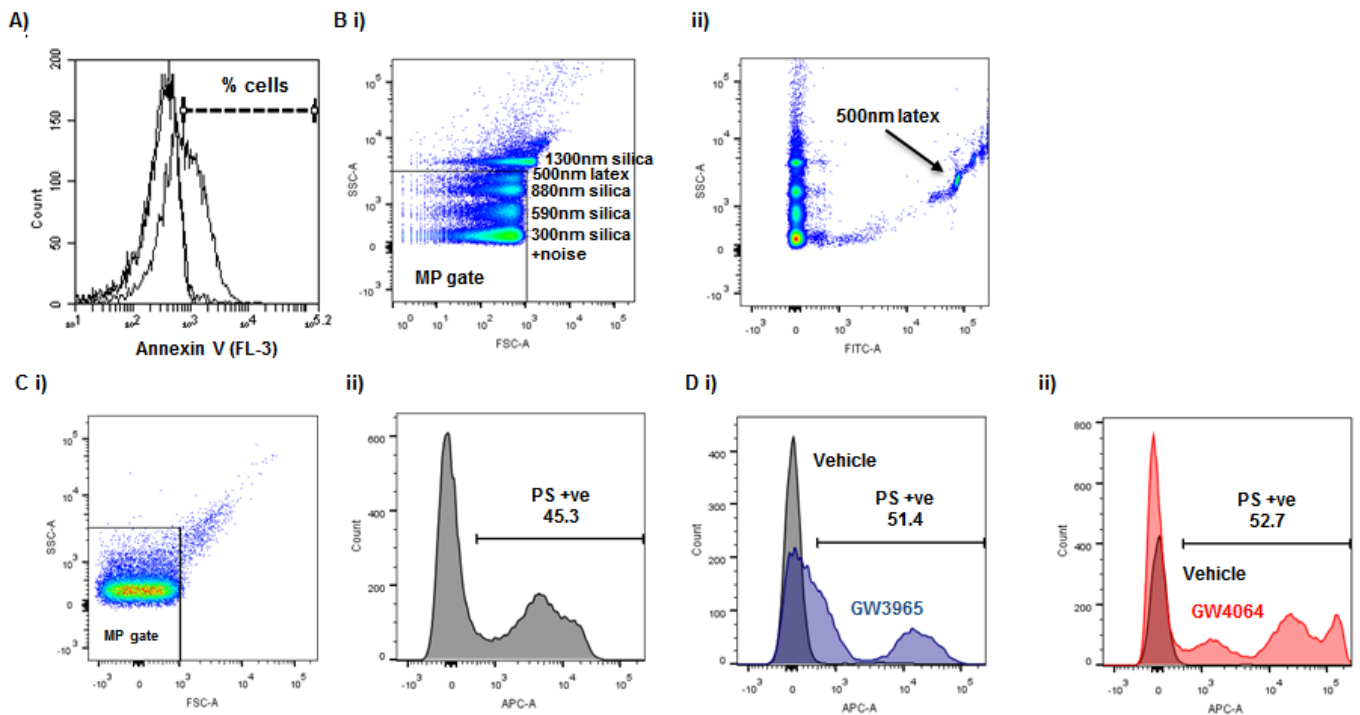
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**FXR and LXR ligand dependent formation of COATED platelets**

**Supplemental figures.**

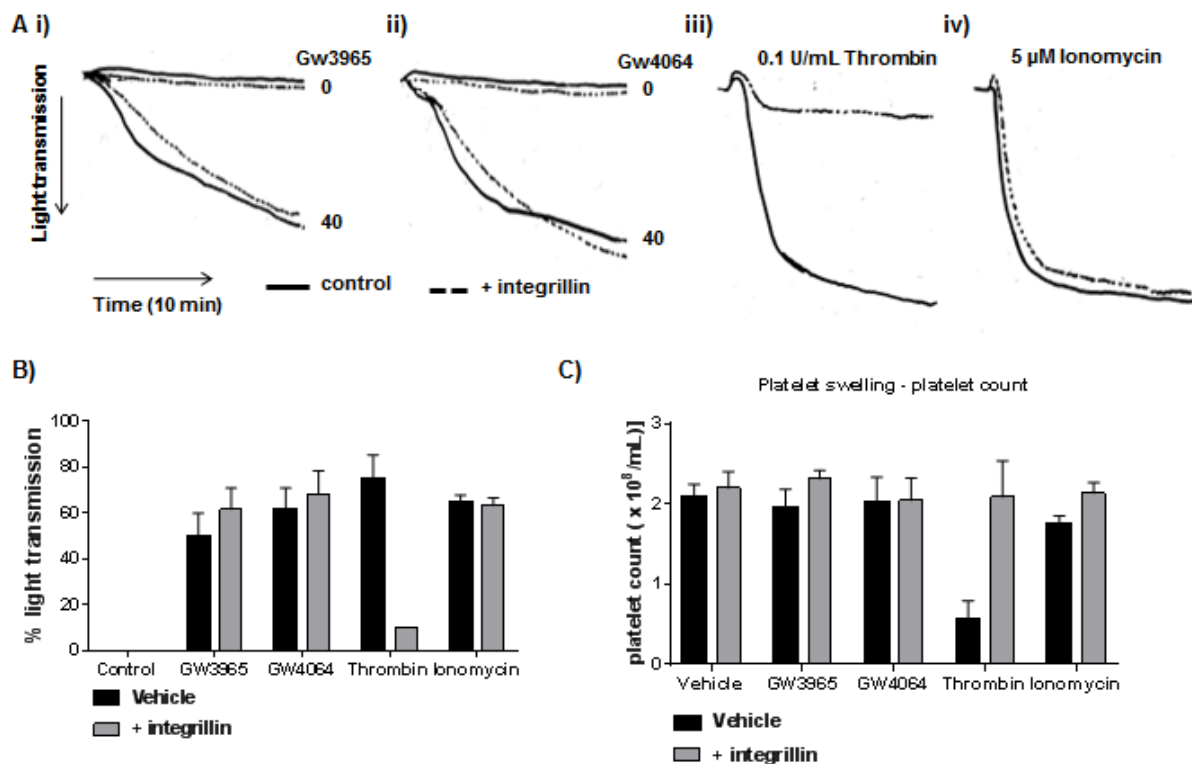
## Supplementary Figure I. LXR and FXR agonists initiate phosphatidylserine exposure and microparticle formation

Human washed platelets were treated for 10 minutes with or without GW3965 (20  $\mu$ M) or GW4064 (20  $\mu$ M) or vehicle control before analysis by flow cytometry for A) Annexin V binding and B and C) Microparticle formation. A) Gating strategy for selecting Annexin V positive cells. B) Microparticle gating strategy shown. Microparticles were determined by gating for the microparticle population using forward and side scatter profiles using a Canto II flow cytometer set up. i) FSC vs. SSC plot of ApogeeMix beads and position of microparticle (MP) gate, set using 500nm latex beads. ii) FITC vs SSC plot confirming the identification of the FITC fluorescent 500nm latex beads. C) Representative i) FSC vs. SSC plots and ii) APC-A fluorescence of PS exposure (Annexin V bound) histograms of platelet derived microparticles following treatment with ionomycin. D) Representative APC-A fluorescence of PS exposure (Annexin V bound) histograms of platelet derived microparticles following treatment with i) GW3965 or ii) GW4064.



## Supplementary Figure II. LXR and FXR agonists initiate platelet swelling.

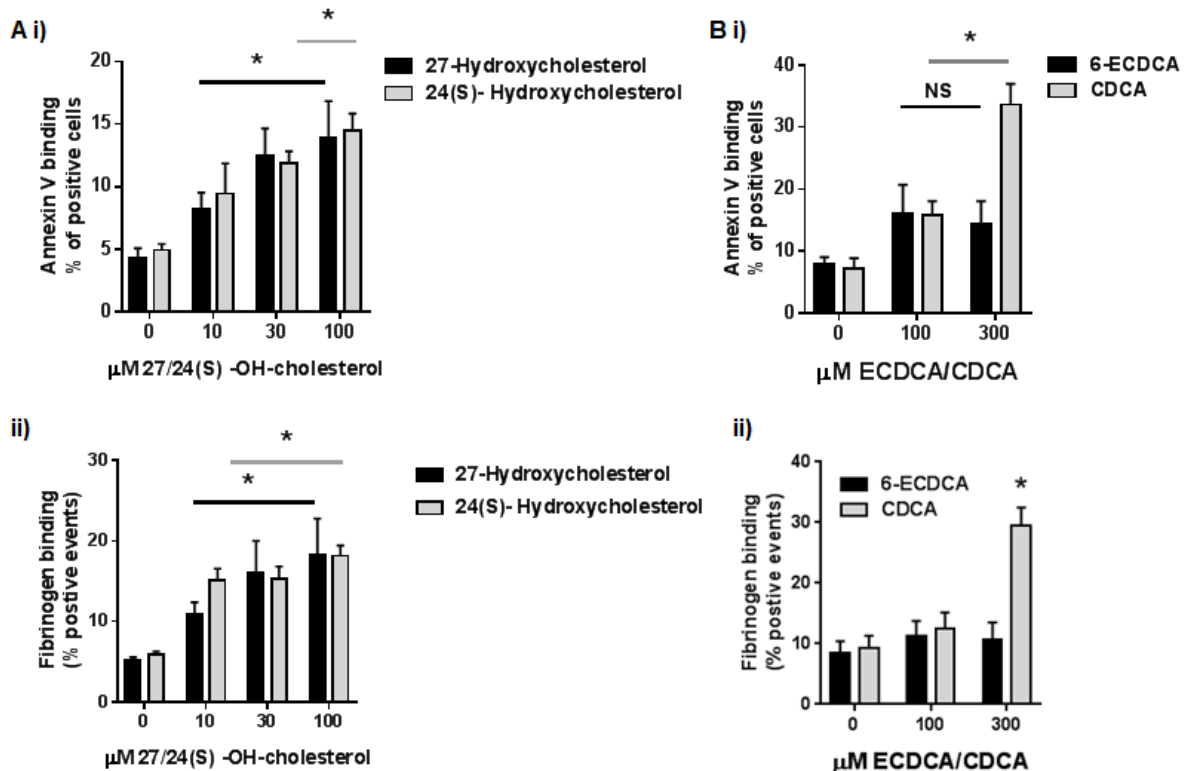
Human washed platelets were treated for 10 minutes with or without i) GW3965 (40  $\mu$ M), ii) GW4064 (40  $\mu$ M), iii) Thrombin (0.1 U/mL), iv) Ionomycin (5  $\mu$ M) or vehicle control in the presence or absence of integrillin (10  $\mu$ M) before platelet swelling measured by analysing light transmission was determined. An increase in light transmission is associated with increased platelet swelling. A) Representative images of light transmission over time, black lines represent in the absence of integrillin, dotted lines represent in the presence of integrillin. B) data expressed as % of light transmission. C) platelet count. Results are mean  $\pm$  S.E.M. for  $n \geq 3$ , \* indicates  $p < 0.05$  in comparison to vehicle controls unless stated otherwise.





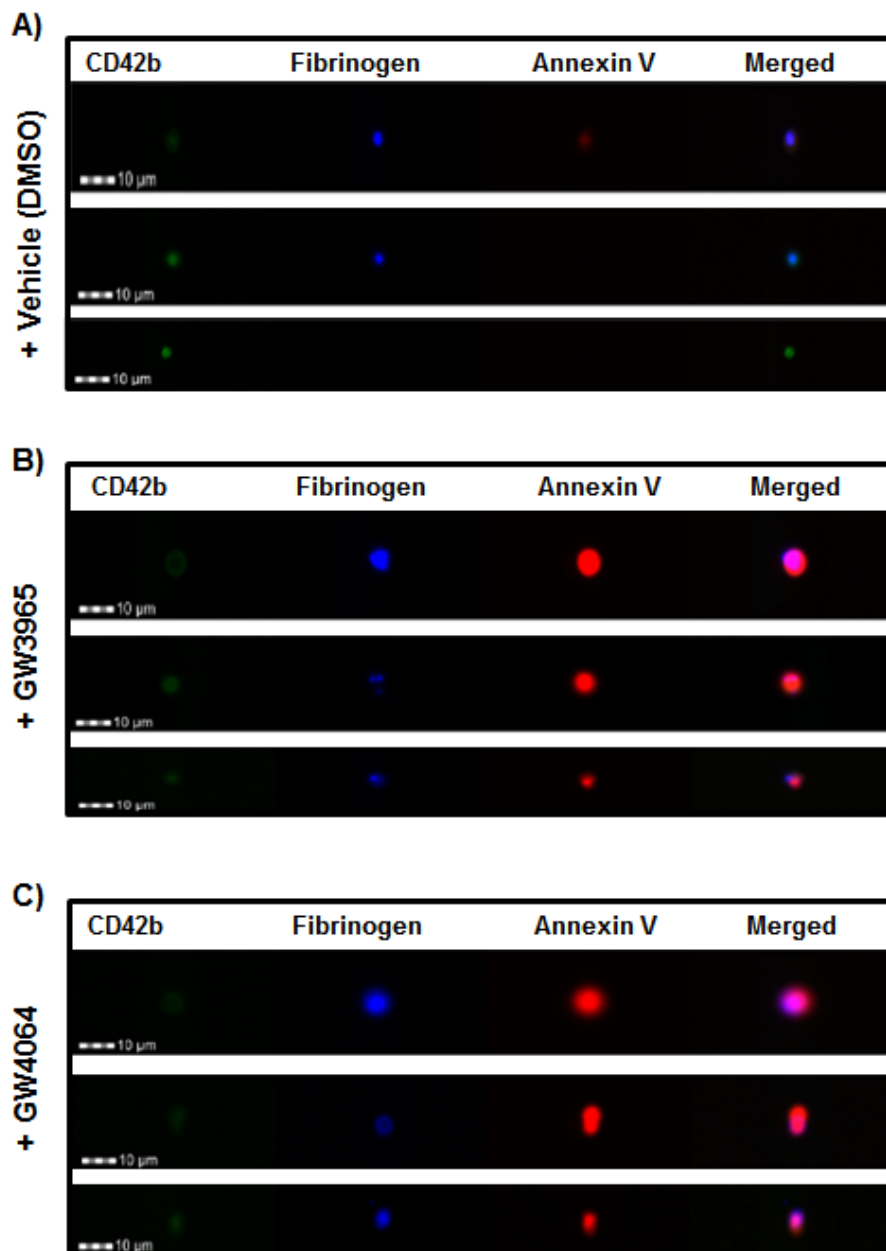
### Supplementary Figure III. LXR and FXR agonists increase Annexin V and fibrinogen binding.

Human washed platelets were treated for 10 minutes with or without increasing concentrations of A) 27-hydroxycholesterol or 24S-hydroxycholesterol (10, 30, 100  $\mu$ M) or B) 6-Ethylchenodeoxycholic acid (6-ECDCA) or chenodeoxycholic acid (CDCA) (100, 300  $\mu$ M) or vehicle control before analysis by flow cytometry for i) Annexin V binding which is a measure of phosphatidylserine exposure or ii) fibrinogen binding, data expressed as percentage of annexin V or fibrinogen positive events. Results are mean + S.E.M. for  $n \geq 3$ , \* indicates  $p < 0.05$  in comparison to vehicle controls unless stated otherwise.



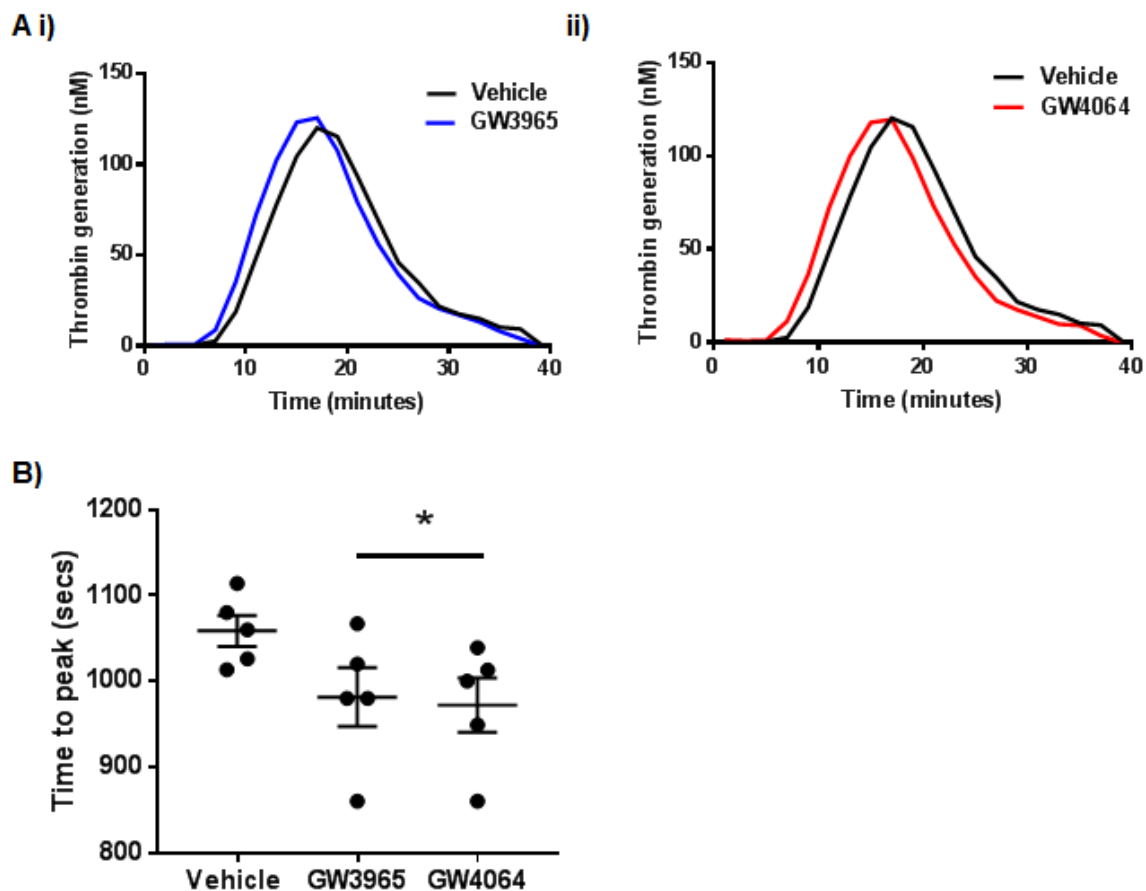
**Supplemental Figure 4. GW3965 and GW4064 increase platelet Annexin V and fibrinogen binding.**

Human washed platelets ( $8 \times 10^7$  cells/ml) were incubated for 20 minutes with fluorescein isothiocyanate-labelled (FITC) anti-fibrinogen (blue), Alexa-647 conjugated anti-Annexin V (red) and PE Cy conjugated anti-CD42b antibodies (green) in the presence of A) vehicle control (DMSO), B) GW3965 (20  $\mu$ M) or C) GW4064 (20  $\mu$ M) and platelets visualised using an ImageStream<sup>x</sup> MarkII imaging flow cytometer. 3 representative images from 3 separate experiments shown.



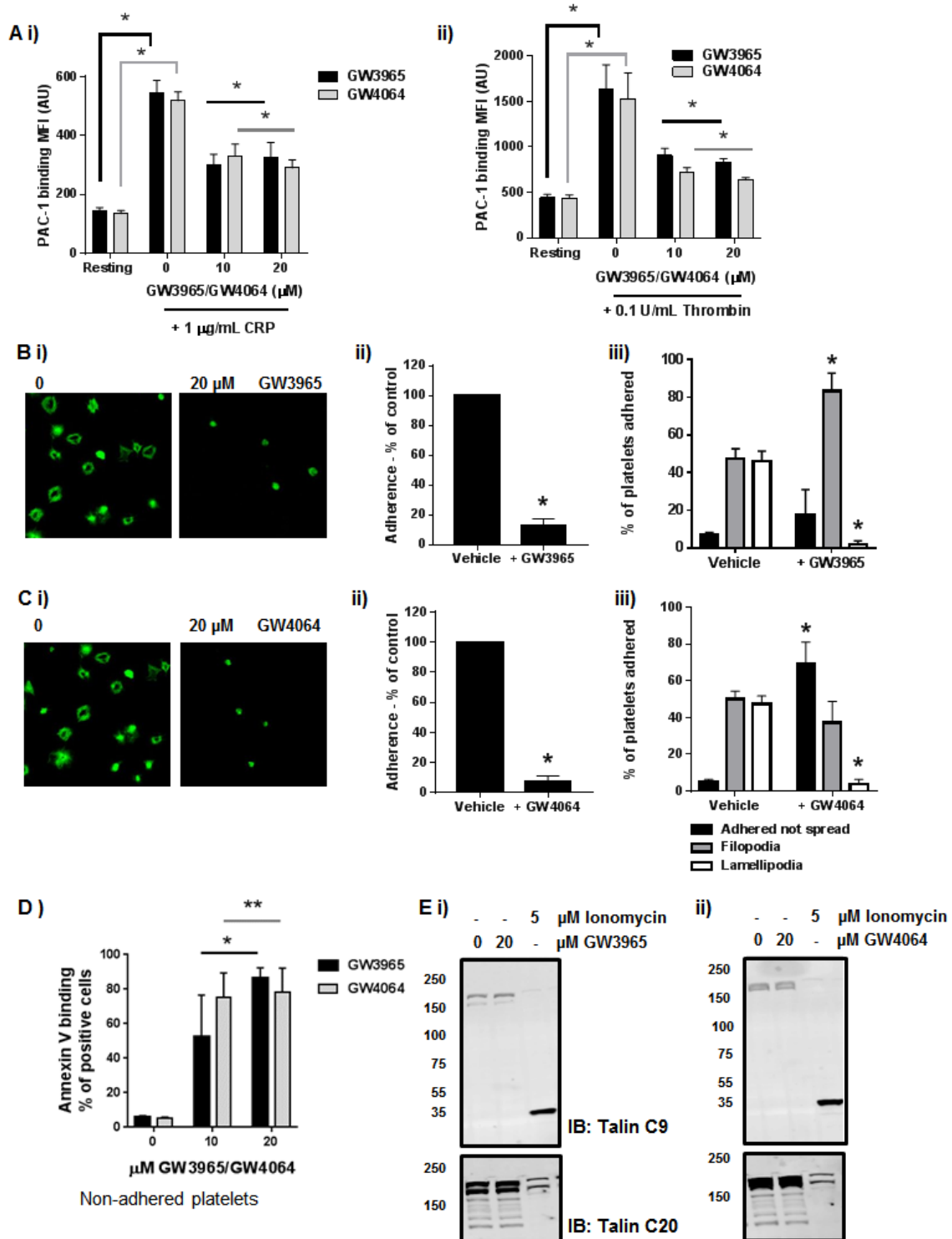
**Supplemental Figure V. GW3965 and GW4064 speed up the kinetics of thrombin generation in platelet rich plasma.**

Calibrated automated thrombography was performed on platelet rich plasma using a tissue factor trigger (final concentration 1 pM) following treatment with, vehicle (0.1 % DMSO), GW3965 (100  $\mu$ M) or GW4064 (100  $\mu$ M) (higher concentrations used to adjust for plasma binding) for 20 minutes before analysis. A) representative traces of thrombin generation over time shown following treatment with vehicle or i) GW3965 or ii) GW4064. B) Time to peak thrombin generation (time from coagulation activation to peak thrombin generation). Results where relevant represent mean  $\pm$  S.E.M. for n=5, \* indicates  $p \leq 0.05$  in comparison to vehicle controls.



## **Supplemental Figure VI. GW3965 and GW4064 inhibit platelet adhesion on fibrinogen.**

Human washed platelets pretreated for 10 minutes with or without GW3965 (10, 20  $\mu$ M), GW4064 (10, 20  $\mu$ M) or vehicle control were A) stimulated with i) 1  $\mu$ g/mL CRP or ii) Thrombin (0.1 U/mL) and then analysed for integrin  $\alpha$ IIb $\beta$ 3 activation by monitoring PAC-1 binding using flow cytometry. Data expressed as median fluorescent intensity. B, C, D) exposed to fibrinogen (100  $\mu$ g/mL) coated coverslips. B, C ) left to adhere and spread on fibrinogen (100  $\mu$ g/mL) coated coverslips. i) Representative images of spreading and adhesion after 45 min in vehicle and treated samples. Platelets were stained with phalloidin Alexa-488 for visualisation. Images were taken under oil immersion with magnification  $\times$ 100. ii) Adhesion: number of platelets adhered were counted in 5 randomly selected fields of view and the number of cells adhered expressed as a percentage of the vehicle treated control. iii) Spreading: platelets were classified into 3 different categories to determine the extent of their spreading (Adhered but not spread, Filopodia: platelets in the process of extending filopodia and Lamellipodia: platelets in the process of extending lamellipodia including those fully spread) at least 100 platelets of each type were scored. Results expressed as relative frequency, as a percentage of the total number of platelets adhered. D) Analysis of Annexin V binding by flow cytometry of non-adhered platelets following exposure to to fibrinogen (100  $\mu$ g/mL) coated coverslips for 45 minutes. E) analysed for talin cleavage (a marker of integrin activation, in platelet lysates was determined by western blotting ), ionomycin (5  $\mu$ M) was included as a positive control. Representative images shown. Results where relevant represent mean + S.E.M. for  $n \geq 3$ , \* indicates  $p \leq 0.05$  in comparison to vehicle controls.





## **FXR and LXR ligand dependent formation of COATED platelets.**

### **MATERIALS AND METHODS**

#### **Reagents**

Nuclear Receptor ligands, GW3965 and GW4064 were purchased from Sigma Aldrich (Poole, UK). BAPTA, DPPD (N-N Diphenyl-p-phenylenediamine), Z-Gly-Gly-Arg-AMC fluorescent substrate and thrombin were purchased from Sigma Aldrich (Poole, UK), ABT-263 from Selleck Chem (Newmarket, UK) and Z-VAD-FMK from BD Biosciences (Oxford, UK). CRP-XL from Prof. R Farndale (University of Cambridge, UK). Fluorescein isothiocyanate-labelled (FITC) anti-fibrinogen antibody was purchased from Dako, PE/Cy5 anti-human CD62P (P-selectin), PE conjugated anti-CD42b antibody and Cy5.5 labelled Annexin V were purchased from BD Biosciences (Oxford, UK). Anti-caspase 3 and cleaved caspase 3 antibodies were purchased from New England BioLabs (Cell Signalling) (Hitchin, UK), 14-3-3 and actin (C20) antibodies were purchased from Santa Cruz Biotechnology, USA, anti-fibrin antibody was a gift from C. Dubois and conjugated to Alexa-647 in house. Fluorophore conjugated secondary antibodies, Alexa-647 labelled Annexin V and Fluo-4 calcium indicator dye were purchased from Life Technologies (Paisley, UK).

#### **Human washed platelet preparation**

Human blood was taken from consenting aspirin free healthy volunteers. Blood was collected into a syringe containing 4% (weight/volume) sodium citrate and acid citrate dextrose (ACD) was added prior to preparation of platelet rich plasma (PRP). Washed platelets were prepared by first isolating the PRP following centrifugation at 100 xg for 20 minutes at room temperature, and centrifugation twice at 1000 xg for 10 minutes to pellet the platelets in the presence of 1.25 µg/mL prostacyclin (PGI<sub>2</sub>) as described previously [1]. Platelets were resuspended in modified Tyrode's-HEPES buffer, (134mM NaCl, 0.34mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9mM KCl, 12mM NaHCO<sub>3</sub>, 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5mM glucose and 1mM MgCl<sub>2</sub>, pH 7.3) and rested for 30 minutes at 30°C before experiments.

#### **Measuring fibrinogen binding, alpha-granule secretion, fibrin binding, membrane integrity and phosphatidylserine exposure by flow cytometry**

Flow cytometry was used to examine affinity up regulation of the integrin αIIbβ<sub>3</sub>, alpha granule secretion, fibrin binding, membrane integrity and phosphatidylserine exposure by detecting levels of fibrinogen binding to integrin αIIbβ<sub>3</sub>, P-selectin exposure on the platelet surface, fibrin bound to the platelet surface, calcein permeability and Annexin V binding respectively in human washed platelets. Following the addition of the nuclear receptor agonists, platelets were incubated at room temperature for 20 minutes with either fluorescein isothiocyanate-labelled (FITC) anti-fibrinogen antibody, PE/Cy5 anti-human CD62P (P-selectin), Alexa-647 conjugated anti-fibrin antibody and Cy5.5 labelled Annexin V or pre loaded with calcein-AM. Antibodies were all used at saturating concentrations, and the instrument settings were kept the same throughout the experiments. Reactions were stopped by a 1 in 5 dilution in 0.2% (v/v) paraformaldehyde or HEPES buffered saline. Flow cytometric acquisition was performed using a BD Accuri C6 flow cytometer, and data were collected from 10,000 events in the platelet gate (determined by Forward and side scatter profiles) and analysed by calculating median fluorescence intensity and percentage of positive cells using CFlow Sampler software.

#### **Flow cytometric analysis of platelet derived microparticles**

Analysis of the generation of microparticles (MP) following treatment of washed platelets ( $2 \times 10^7$  /mL) with GW3965, GW4064 or Ionomycin was carried out by flow cytometry, using a BD Canto II Flow Cytometer (BD Biosciences, Oxford UK) set to trigger on side scatter (SSC) and all data were analysed using FlowJo software. ApogeeMix beads (Apogee Flow Systems, Hemel Hempstead UK), comprised of 180nm, 240nm, 300nm, 590nm, 880nm and 1300nm silica beads and 110nm and 500nm green fluorescent latex beads, were used to determine the optimum SSC and forward scatter (FSC) voltages for microparticle detection. The microparticle gate was set using the 500nm latex beads (Supplemental Fig 1B) <sup>[2]</sup>. To acquire data, events were collected over 2 mins and a BD Trucount tube was run at the end of each session to measure the volume of sample analysed. Prior to running samples, Modified-Tyrodes- HEPEs buffer was also run for 2 mins to assess the level of background events. Prior to use, annexin V conjugated to APC (Ann V-APC; Life Technologies) was filtered using Millipore 0.1µm centrifugal devices (Merck Millipore, Feltham UK) to minimize interference by background microparticles. Ann V-APC was also titrated to ensure use at the optimum concentration. Modified-Tyrodes- HEPEs buffer and annexin V-APC (4 µL) alone controls were run to measure background contaminating events. Unlabeled samples were also run to set the gate for annexin V-APC positive microparticles to include  $\leq 1\%$  of background events. Samples were then incubated with annexin V-APC (1:200 dilution) for 15min at R/T in the dark and then acquired for 2 min on the flow cytometer. The number of annexin V positive events in the GW3965 and GW4064 treated sample was then counted following gating using a vehicle treated annexin v-APC labelled control for comparison.

### **Platelet swelling assay**

Washed platelets were prepared from human blood and platelet swelling measured by optical transmission in an aggregometer (Chronolog Corp., Havertown, PA, USA) as described previously <sup>[2]</sup>. An increase in light transmission without aggregate formation and alteration in platelet count is indicative of platelet swelling. Platelet counts were monitored after treatment to confirm lack of cell lysis or aggregation <sup>[2]</sup>. Mean platelet volume was determined by using an ACT5 haematology analyser (Beckman Coulter Inc, UK).

### **Measuring mitochondrial membrane potential by flow cytometry.**

The integrity of platelet mitochondrial membranes was measured in washed platelets preloaded with the fluorescent dye JC-1 and the FL1/FL2 ratio for each sample measured by flow cytometry. Samples were prepared as described in the earlier flow cytometry section. Flow cytometric acquisition was performed using a BD Accuri C6 flow cytometer, and data were collected from 10,000 events in the platelet gate (determined by Forward and side scatter profiles) and analysed using CFlow Sampler software. An increase in the ratio compared to control indicates hyperpolarisation of the mitochondrial membrane whilst a decrease in the FL2/FL1 ratio indicates membrane depolarisation <sup>[3]</sup>.

### **Single platelet $\text{Ca}^{2+}$ imaging.**

PRP was loaded with Fluo-4 AM (2 µM) for 1h at 30°C and then washed by centrifugation at 350 xg for 20 mins and resuspended in Tyrode's-HEPEs buffer in the presence of 1mM  $\text{CaCl}_2$ . Platelets were then attached to mouse anti-human PECAM-1 antibody (WM59) coated glass-bottom Vena8 GCS biochips (Cellix Ltd., Dublin, Ireland), which had been pre-incubated with 2% BSA for 1 hour at 37°C to prevent glass-induced platelet activation. Vehicle, GW3965 (20µM) or GW4064 (20 µM) were then flowed through the chips at low shear rate ( $400 \text{ s}^{-1}$ ) and calcium signalling monitored by observing fluorescence measurements with excitation at 488 nm and emission at 525 nm with a 60x magnification lens using a Nikon A1-R confocal microscope.

### **Immunofluorescence microscopy.**

Human platelets treated with or without GW3965 (20 $\mu$ M) or GW4064 (20  $\mu$ M) and fixed in 0.2% formalin were left to settle on poly-L-lysine coverslips for 1 hour at 37°C before blocking (1% BSA, 2% donkey serum). Coverslips were then incubated with an Alexa 488nm fluorophore conjugated anti-fibrinogen antibody and Cy5.5 labelled Annexin V for 1 hour at room temperature in the dark. Coverslips were washed and mounted onto slides. Platelets were imaged with a 100 x magnification oil immersion lens on a Nikon A1-R confocal microscope. Data analysed using ImageJ software.

### **Imaging flow cytometry**

For additional imaging of coated platelet formation, an ImageStream<sup>×</sup> Mark II imaging flow cytometer (Amnis Corporation, WA, USA) was used because this combines flow cytometry with high content image analysis. To prepare samples, washed human platelets (8x10<sup>7</sup> cells/ml) were pre-treated with vehicle (0.1% DMSO), GW3965 (20  $\mu$ M) or GW4064 (20  $\mu$ M) for 20 minutes and stained with anti-CD42b-PE to enable detection of the platelet population, anti-fibrinogen- FITC and annexin-V conjugated to Alexa-647 and then diluted 10-fold in modified Tyrodes buffer. The ImageStream<sup>×</sup> Mark II imaging flow cytometer was then used to acquiring up to 12 images of cell including, brightfield, scatter, and multiple fluorescent images. Briefly, the platelet population was gated by size and PE- CD42b positivity and confirmed visually by acquired images. Data for 10 000 events were collected and analysed using IDEAS software (Amnis, Seattle, WA)

### **Serine protease activity assay**

Human washed platelets were preincubated with Z-Gly-Gly-Arg-AMC fluorescent substrate prior to addition of nuclear receptor agonists, using a 96 well plate assay approach and fluorescence plate reader. Increases in levels of fluorescence as a result of serine protease activity and cleavage of the AMC group was monitored at 37°C for 1 hour at an excitation wavelength of 366nm and emission wavelength of 460nm.

### **Thrombin generation assay**

Calibrated automated thrombography was performed on platelet rich plasma using a tissue factor trigger (final concentration 1 pM). Vehicle (0.1% DMSO), GW3965 (100  $\mu$ M) or GW4064 (100  $\mu$ M) was added to platelet rich plasma 20 minutes before analysis. Coagulation was initiated with calcium chloride and was continuously registered using a fluorogenic substrate (ZGGR-AMC; FluCa reagent). Thrombin generation was measured against a thrombin calibrator using an automated fluorometer (Fluoroskan Ascent®, ThermoLabsystem, Helsinki, Finland) and output was analysed using Thrombinscope software (Thrombinscope BV).

### **Immunoblotting**

SDS-PAGE and immunoblotting were performed using standard techniques as described previously <sup>[1]</sup>. Proteins were detected using antibodies raised against the protein of interest and fluorophore-conjugated secondary antibodies and then visualised using a Typhoon imaging system (GE Healthcare).

### **Statistical analysis**

Statistical analyses were carried out on data using GraphPad prism software. When comparing two sets of data, an unpaired, 2-tailed Student's t test was used. If more than two means were present, significance was determined by one way ANOVA. Where data is normalised, statistical analysis was performed prior to normalisation and also using the non-

parametric Wilcoxon signed-rank test.  $P \leq 0.05$  was considered statistically significant. Unless stated otherwise, values are expressed as mean  $\pm$ SEM.

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2. Mattheij NJ, Gilio K, van Kruchten R, et al., Dual mechanism of integrin  $\alpha$ IIb $\beta$ 3 closure in procoagulant platelets. *J Biol Chem*, 2013; 288(19):13325-36.
3. Verhoeven AJ, Verhaar R, Gouwerok EG, and de Korte D, The mitochondrial membrane potential in human platelets: a sensitive parameter for platelet quality. *Transfusion*, 2005; 45(1):82-9.